The application has been amended, in accordance with the Examiner's suggestion, to incorporate the nucleic acid and amino acid sequences of the III2R and H2F antibodies, both of which are disclosed in the Manheimer-Lory paper. The enclosed Amendment Under 35 C.F.R. §1.825 (a) and (b) inserts additional sequence listings into the application and this paper inserts SEQ ID NOS into the specification at the relevant points. Manheimer-Lory is cited in the specification (page 36, lines 4-20) and is incorporated by reference (page 54, lines 23-24). The amendment therefore does not constitute new matter. Applicants verify that the sequences disclosed in the Manheimer-Lory paper are the same sequences recited in this amendment. *See In re Hawkins,* 486 F.2d 569-576, 179 U.S.P.Q. 157-162 (C.C.P.A.1973) (holding that essential matter originally cited in a foreign patent and incorporated by reference could be added by amendment and did not constitute new matter where accompanied by a declaration regarding accuracy of the amendment).

No new matter has been added by this amendment. This amendment does not narrow the scope of any claim.

Objection Under 35 U.S.C. § 132 and 35 U.S.C. § 112

The 3D1 Antibody

In a Supplemental Amendment dated October 15, 2002 Applicants submitted the entire amino acid and nucleotide sequence of 3D1 antibody thereby making the antibody available to the public. The Office has now objected to this amendment, alleging the application as filed does not provide support for the entire nucleotide and

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

amino acid sequence. According to the Office, the specification only provides support for the sequence of the variable region. Applicants respectfully submit that the Office has misunderstood the specification.

Disclosure of the 3D1 antibody is not limited to merely the variable region, as suggested by the Office. For example, the specification states "the terms 'HF2.3D1' and '3D1' refer to murine immunoglobulin specific to B7-2. . . . The terms 'immunoglobulin' or 'antibody' include whole antibodies. . . " (page 14, lines 1-4). The specification also states on page 4 lines 1-7 (emphasis added):

> In particular an embodiment of the invention is a humanized immunoglobulin which specifically binds to B7-2 and comprises a humanized light chain comprising three light chain CDRs from the mouse 3D1 antibody and a light chain variable region framework sequence from a human immunoglobulin light chain, and a humanized heavy chain comprising three heavy chain CDRs from the mouse 3D1 antibody and a heavy chain variable region framework sequence from a human immunoglobulin heavy chain

The term, comprising, is open ended and therefore would include portions of the 3D1 antibody outside of the variable region. The specification therefore supports the entire sequence of the antibody and Applicants respectfully submit the Office's objection is unfounded. Additionally, Applicants now submit a sequence listing of the 3D1 antibody in conformance with 35 C.F.R. §1.825(a)(b). Thus, Applicants request that the Office withdraw this rejection.

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

Enablement Rejection Under 35 U.S.C. § 112 First Paragraph

Claims 1-4 and 6-26 stand rejected under 35 U.S.C. §112, first paragraph for alleged lack of enablement. The Office maintains the rejection alleging the specification fails to disclose either an appropriate biological deposit or a disclosure of the entire sequence of the recited antibodies. For the reasons put forth below Applicants submit the rejection is in error.

The Claims Are Enabled Without Recitation of SEQ ID NOS

Because the claims recite "3D1," "H2F," and "III2R," the Office alleges that the claims must also recite either the appropriate deposit information (Accession Numbers) and/or SEQ ID NOS for the "3D1," "H2F," and "III2R" antibodies to satisfy the enablement requirement under 35 U.S.C. § 112. Applicants traverse and respectfully submit the claims meet the enablement requirement.

"The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation." *United States v. Telectronics, Inc.* 857 F. 2d 778, 785, 8 U.S.P.Q. 2d 1217,1223 (Fed. Cir. 1988). The sequences of the three antibodies are known in the art and disclosed in the specification. A skilled artisan armed with the sequences disclosed in the specification and known in the art would be able to practice the claimed invention without any undue experimentation. Thus, there would be no need to recite the SEQ ID NOS of the three antibodies. Resort to the specification would provide the skilled artisan with adequate guidance to practice the

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

invention. Nonetheless, in order to expedite prosecution Applicants have amended the claims to recite the SEQ ID NOS of the 3D1 antibody and the variable region of the III2R and H2F antibodies.

The 3D1 Antibody

The Office acknowledges that the entire nucleotide and amino acid sequence of the 3D1 antibody has been submitted. The Office has stated disclosure of the entire amino acid sequence would satisfy the enablement requirement, (Paper 19, Office Action dated December 30, 2002). Thus, with respect to the 3D1 antibody Applicants submit the claims are enabled.

The III2R and H2F Antibodies

The Office has acknowledged that Applicants never actually used the III2R or H2F antibodies in practicing the claimed invention, but rather merely relied on portions of the printed sequences to derive the claimed subject matter. The Office has also acknowledged the availability of the sequences for the III2R and H2F antibodies in the prior art. The Office, however, objects to the recitation of these antibodies in the claims because according to the Office the antibodies are essential subject matter which cannot be incorporated by reference. Without conceding the correctness of the objection, and for the sole purpose of expediting prosecution, Applicants have amended the specification herein to recite the nucleic acid and amino acid sequences of the III2R (SEQ ID NOS: 25, 27, 29, 31) and H2F (SEQ ID NOS: 26, 28, 30, 32) variable domains

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

as disclosed in Manheimer-Lory, *J. Exp. Med.* 174:1639 (1991). In doing so, Applicants state that the sequence disclosed in Manheimer-Lory is the same sequence incorporated into the specification by amendment made herein.

In making this amendment Applicants do not concede the correctness of the rejection. Applicants maintain the claimed invention is enabled by the specification. Applicants submit that the basis of this rejection has no merit because the Office has mischaracterized the claims. According to the Office the claims recite as "reference or starting materials" the III2R and H2F antibodies. This is incorrect. The claims only recite small portions of these antibodies (e.g. the framework region of the variable domain). Because the claims only recite the variable region of these antibodies, disclosure of the entire antibody sequence is not required to practice the claimed invention.

The sequences of the variable domains were known in the art and disclosed in the specification by virtue of the citation to the Manheimer-Lory paper. The sequences of these domains are short (*see* figure 2 and Manheimer-Lory already of record) and thus, could be easily synthesized without any undue experimentation. Oligonucleotide synthesis was known in the art (*see e.g.,* S. L. Beaucage and M. H. Caruthers, 1981, *Tetrahedron Lett.* 22:1859) (courtesy copy enclosed). Moreover, humanizing antibodies using oligonucleotide synthesis of framework and CDR regions was practiced in the art (*See e.g.*, U.S. Patent No. 5,585,089, at column 22, lines 25-35) (already of record) and more importantly, was taught in the instant specification (page 24, line 3-page 25, line 2). A skilled artisan could easily practice the claimed invention without undue

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

experimentation based on the disclosure in the specification and the teaching of the prior art. The invention as now claimed is thus enabled.

Indefiniteness Under 35 U.S.C. § 112 Second Paragraph

The 3D1, H2F and III2R Antibodies

Claims 1-4 and 6-26 stand rejected as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which the Applicants regard as the invention. According to the Office, 3D1, H2F, and III2R are merely "laboratory designations" which do not clearly define the claimed invention. Applicants submit the rejection has no basis.

The purpose of the definiteness requirement is to inform the public of the boundaries of infringing conduct. MPEP § 2173. A fundamental principle contained in 35 U.S.C. § 112, second paragraph is that Applicants are their own lexicographers. MPEP § 2173.01 "Definiteness of claim language must be analyzed, not in a vacuum, but in light of (A) the content of the particular application disclosure; (B) the teachings of the prior art; and (C) the claim interpretation that would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made." MPEP §2173.02 (emphasis added). When all of these factors are considered the claim terms cannot be deemed indefinite.

The content of the specification coupled with the teaching of the prior art make the terms 3D1 antibody and H2F and III2R variable regions definite. The sequences of each one of the 3D1 antibody and H2F and III2R variable regions were known in the art.

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

References disclosing the sequences are cited in the specification. (See *e.g.,* page 36, lines 14-17; page 17, lines 1-5). Moreover, at the Examiner's suggestion these sequences have been incorporated into the specification.

A skilled artisan reading the claims in light of the specification would understand III2R, H2F and 3D1 refer to the antibodies or variable regions, respectively, corresponding to the sequences disclosed and cited in the specification. The claim terms, therefore are not indefinite. Moreover, there is nothing of record to the substantiate the allegation that the claim terms are indefinite. The Office merely suggests that there might be other antibodies with the same name. The Office also suggests hybridomas can undergo changes resulting in "microheterogeneity." First, Applicants note both of these suggestions are completely unsubstantiated by the record. Applicants call upon the Examiner to cite a reference to support his position or alternatively provide an affidavit pursuant to 37 C.F.R. §1.104(d)(2). Absent such a demonstration, the claimed invention when considered in light of the specification satisfies the definiteness requirement. Secondly, Applicants submit that if the Office's concerns regarding "microheterogeneity" are accurate any deposited hybridoma would, by necessity, be indefinite with regard to the antibody it produces. Thus a deposited hybridoma could never satisfy 35 U.S.C. § 112. Clearly, this is not the case. (*see* 37 C.F.R. § 1.801; MPEP § 2403) Nonetheless, in order to expedite prosecution Applicant's have amended the claims to recite the SEQ ID NOS of the respective antibodies thus obviating this rejection.

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

The Rejection Under 35 U.S.C. § 103(a)

Claims 1-12, 15, 21, 23-25, 27, 28, 30-36, 38-40, 46 and 64-76 stand rejected under 35 U.S.C. §103 as being obvious and thus unpatentable over U.S. Patent No. 6,084,067 (Freeman) in view of the art recognized methods of cloning and expressing recombinant antibodies, as evidenced by U.S. Patent No. 5,585,089 (Queen) and *Antibodies: A Laboratory Manual,* Chapter 3, Cold Spring Harbor Laboratory, 1988, (Harlow and Lane Eds.) (Harlow). The Office alleges Freeman teaches humanized antibodies to B7-2, Queen teaches improved methods of humanizing antibodies which maintain binding affinities of at least about 10^8 M^{-1}, and Harlow teaches $10^7 M^{-1}$ is a weak affinity for an antibody. The Office concludes that the claimed invention is allegedly obvious in light of the prior art. Applicants respectfully traverse the rejection.

The Patent Office bears the burden of establishing the claimed invention is prima facie obvious. MPEP § 2142. The PTO has not met its burden in the instant case.

The Claimed Invention Is Not Prima Facie Obvious

MPEP § 2143 provides the standard required to establish a prima facie case of obviousness. "First there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one or ordinary skill in the art, to modify the reference or to combine what the reference teaches. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references combined) must teach or suggest all the claim limitations."

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

The motivation to make the claimed invention and the reasonable expectation of success must both be found in the prior art, not the applicant's disclosure. *In re Vaeck,* 947 F.2d 488, 493, 20 U.S.P.Q.2d 1438, 1442 (Fed. Cir. 1991). The references must be considered as a whole and must suggest the desireability, and thus the obviousness, of making the combination. *Hodosh v. Block Drug Col, Inc.,* 786 F.2d 1136, 1143 n.5, 229 U.S.P.Q. 182, 187 n.5 (Fed. Cir. 1986); MPEP § 2141.

The Cited Art Does Not Teach All Of The Claim Limitations

The prior art references combined do not teach all of the claim limitations. The claims now amended recite "a humanized immunoglobulin comprising: . . . at least a portion of an immunoglobulin of human origin derived from the III2R and/or the H2F variable region, . . . and the humanized immunoglobulin has a binding affinity of at least about 10^7M^{-1}." The Examiner has cited three references, Freeman, Queen, and Harlow to support the obviousness rejection. Harlow does not teach humanization of antibodies, but teaches antibodies have a wide range of affinities. Freeman suggests the possibility of making humanized B7-2 specific antibodies, but does not teach specific affinities. Queen discloses humanized antibodies with affinities greater than 10^7M^{-1} that recognize various epitopes (e.g. IL-2 receptor and various herpes virus proteins), but does not disclose B7-2 specific antibodies. None of these references disclose an antibody with at least a portion of the amino acid sequence of the variable region in common with at least a portion of the amino acid sequence of the variable region of either the III2R or H2F antibody. Because the combined references relied on

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

do not teach or suggest each claim limitation the office has not established prima facie that the claimed invention is obvious.

The Cited References Fail To Provide A Reasonable Expectation of Success

Even if the skilled artisan had been aware of the III2R and H2F antibodies, he or she would have had no reasonable expectation of success in using their sequences to modify the 3D1 antibody.

The claimed invention derives the heavy chain framework from the III2R antibody and the light chain framework from the H2F antibody. The CDRs are derived from the 3D1 antibody. The Examiner has previously pointed to Freeman as disclosing the use of the 3D1 antibody against B7-2. But nothing in the Freeman or any cited reference suggested that homology existed between the 3D1 antibody and the variable region of either the H2F or III2R antibodies. The Examiner is reminded the desirability and reasonable expectation of success must be found in the prior art, and not based on hindsight in light of the Applicant's disclosure. *Vaeck, supra.*

Homology between the CDR donor antibody and the frame work acceptor antibody is critical for success in the humanization process. The specification teaches: "in a preferred embodiment, the FRs of a humanized variable region having at least about 60% overall sequence identity, and preferably at least about 80% overall sequence identity, with the variable region of the nonhuman donor" (page 30, lines 13-16). The cited art also teaches the importance of homology between the donor and acceptor antibodies (*see* Queen, column 13, lines 37-41). Nothing in the art suggested

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

the three antibodies possessed the requisite homology. Without realizing that homology existed between the variable regions of the III2R, H2F and 3D1 antibodies, the skilled artisan would not even contemplate using the variable regions of the H2F and III2R antibodies to humanize the murine 3D1. The art, thus provided no motivation to use the variable regions of the H2F or III2R antibodies and no reasonable expectation of success that the combination would be successful.

Because there was no reasonable expectation of success in attaining the claimed invention based upon the disclosures in the references of record, the claimed invention is not prima facie obvious. Accordingly, Applicants respectfully request withdrawal of the rejection.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants respectfully request the reconsideration and reexamination of this application and the timely allowance of the pending claims.

Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: March 11, 2003

By: E Stewart Mittler
E. Stewart Mittler
Reg. No. 50,316

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

APPENDIX OF AMENDMENTS
VERSION WITH MARKINGS TO SHOW CHANGES

The specification will read as follows:

Please replace the paragraph beginning on page 3[6]5, line [4]1 with the following paragraph:

> To retain the binding affinity of the mouse antibody in the humanized antibody, the general procedures of Queen *et al.* were followed (Queen *et al. Proc. Natl. Acad. Sci. USA* 86: 10029 (1989), U.S. Patent Nos. 5,585,089 and 5,693,762, the teachings of which are incorporated herein in their entirety). The choice of framework residues can be critical in retaining high binding affinity. In principle, a framework sequence from any human antibody can serve as the template for CDR grafting; however, it has been demonstrated that straight CDR replacement into such a framework can lead to significant loss of binding affinity to the antigen (Tempest *et al., Biotechnology* 9: 266 (1992); Shalaby *et al., J. Exp. Med.* 17: 217 (1992)). The more homologous a human antibody is to the original murine antibody, the less likely the human framework will introduce distortions into the mouse CDRs that could reduce affinity. Based on a sequence homology, III2R (SEQ ID NOS:[45] 25, 29) was selected to provide the framework for the humanized 3D1 heavy chain and H2F (SEQ ID NOS:[46] 26, 30) for the humanized 3D1 light chain variable region. Manheimer-Lory, A. *et al., J. Exp. Med.* 174(6):1639-52 (1991). Other highly homologous human antibody chains would also be suitable to provide the humanized antibody framework, especially kappa light chains from human subgroup 4 and heavy chains from human subgroup 1 as defined by Kabat.

The claims will read as follows:

1. (Amended) A method of inhibiting the interaction of a first cell bearing a B7-2 receptor with a second cell bearing B7-2, comprising contacting said second cell

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

with an effective amount of a humanized immunoglobulin having binding specificity for B7-2, said immunoglobulin comprising:

a) at least one antigen binding region of nonhuman origin and

b) at least a portion of an immunoglobulin of human origin derived from the III2R (SEQ ID NOS: 25, 29) and/or the H2F [antibody] SEQ ID NOS: 26, 30) variable region,

wherein the immunoglobulin has at least a portion of the amino acid sequence of its variable region in common with at least a portion of the amino acid sequence of the variable region of the III2R (SEQ ID NOS: 25, 29) and/or the H2F SEQ ID NOS: 26, 30) antibody and the humanized immunoglobulin has a binding affinity of at least about 10^7 M^{-1}.

2. (Amended) A method of inducing immunotolerance in a patient having a transplanted organ, tissue, cell, or the like comprising administering an effective amount of a humanized immunoglobulin having binding specificity for B7-2, said immunoglobulin comprising:

a) at least one antigen binding region of nonhuman origin, and

b) at least a portion of an immunoglobulin of human origin derived from the III2R (SEQ ID NOS: 25, 29) and/or the H2F [antibody] (SEQ ID NOS: 26, 30) variable region,

wherein the immunoglobulin is administered in a carrier, and wherein the immunoglobulin has at least a portion of the amino acid sequence of its variable region in common with at least a portion of the amino acid sequence of the variable region of the III2R (SEQ ID NOS: 25, 29) and/or the H2F (SEQ ID NOS: 26, 30) antibody and the humanized has a binding affinity of at least about 10^7 M^{-1}.

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

3. (Amended) A method of reducing transplantation rejection in a patient having a transplanted organ, tissue, or cell, comprising administering a therapeutically effective amount of a humanized antibody having binding specificity for B7-2, said immunoglobulin comprising:

a) at least one antigen binding region of nonhuman origin, and

b) at least a portion of an immunoglobulin of human origin derived from the III2R and/or the H2F [antibody] variable region,

[wherein the humanized immunoglobulin has a binding affinity of at least about 10^7 M^{-1}.] wherein the immunoglobulin has at least a portion of the amino acid sequence of its variable region in common with at least a portion of the amino acid sequence of the variable region of the III2R (SEQ ID NOS: 25, 29) and/or the H2F (SEQ ID NOS: 26, 30) antibody and the humanized immunoglobulin has a binding affinity of at least about 10^7 M^{-1}.

6. (Amended) The method of claim 1, wherein said at least one antigen binding region further comprises at least one CDR of the variable region of the 3D1 (SEQ ID NOS: 21, 23) antibody.

10. (Amended) The method of claim 1, wherein said [portion] immunoglobulin has at least a portion of the amino acid sequence of its variable region in common with at least a portion of the amino acid sequence of the variable region of the III2R antibody (SEQ ID NOS: 25, 29).

11. (Amended) The method of claim 1, wherein said [portion] immunoglobulin has at least a portion of the amino acid sequence of its variable region in common with

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

at least a portion of the amino acid sequence of the variable region of the H2F antibody (SEQ ID NOS: 26, 30).

12. (Amended) The method of claim 2, wherein said at least one antigen binding region further comprises at least one CDR of the variable region of the variable region of the 3D1 antibody (SEQ ID NOS: 21, 23).

16. (Amended) The method of claim 2, wherein said [portion] immunoglobulin has at least a portion of the amino acid sequence of its variable region in common with at least a portion of the amino acid sequence of the variable region of the III2R antibody (SEQ ID NOS: 25, 29).

17. (Amended) The method of claim 2, wherein said [portion] immunoglobulin has at least a portion of the amino acid sequence of its variable region in common with at least a portion of the amino acid sequence of the variable region of the H2F antibody (SEQ ID NOS: 26, 30).

18. (Amended) The method of claim 3, wherein said at least one antigen binding region further comprises at least one CDR of the variable region of 3D1 antibody.

22. (Amended) The method of claim 3, wherein said [portion] immunoglobulin has at least a portion of the amino acid sequence of its variable region in common with at least a portion of the amino acid sequence of the variable region of the III2R antibody SEQ ID NOS: 25, 29).

23. (Amended) The method of claim 3, wherein said [portion] immunoglobulin has at least a portion of the amino acid sequence of its variable region in common with

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

at least a portion of the amino acid sequence of the variable region of the H2F antibody

SEQ ID NOS: 26, 30).

FINNEGAN
HENDERSON
FARABOW
GARRETT &
DUNNER LLP

1300 I Street, NW
Washington, DC 20005
202.408.4000
Fax 202.408.4400
www.finnegan.com

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Vol 22 No 20 1981

Tetrahedron Letters

The International Journal for the Rapid Publication of Preliminary Communications in Organic Chemistry

Contents

(continued on back cover)

TETRAHEDRON LETTERS

The International Journal for the Rapid Publication of Preliminary Communications in Organic Chemistry

Publishing, Subscription and Advertising Offices

Headington Hill Hall, Oxford OX3 0BW (phone Oxford 64881; telex 83177). Maxwell House, Fairview Park, Elmsford, NY 10523, USA (phone 914 592 7700; telex 137328).

Fifty-two issues per year

Annual Subscription Rates (1981)

For libraries, university departments, government laboratories, industrial and other multiple-reader institutions US $775.00 per annum (including postage and insurance). Two year rate (1981–82) US $1472.50.

Specially Reduced Rates to Individuals

In the interests of maximising the dissemination of the research results published in this important international journal we have established a two-tier price structure. Any individual whose institution takes out a library subscription may purchase a second or additional subscription for personal use at the much reduced rate of US $95.00.

Microform Subscriptions and Back Issues

Back issues of all previously published volumes are available in the regular editions and on microfilm and microfiche. Current subscriptions are available on microfiche simultaneously with the paper edition and on microfilm on completion of the annual index at the end of the subscription year.

Tetrahedron Letters, Vol.22, No.20, pp 1859 - 1862, 1981
Printed in Great Britain

0040-4039/81/201859-04$02.00/0

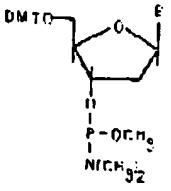
DEOXYNUCLEOSIDE PHOSPHORAMIDITES—A NEW CLASS OF KEY INTERMEDIATES FOR DEOXYPOLYNUCLEOTIDE SYNTHESIS

S. L. Beaucage and M. H. Caruthers*

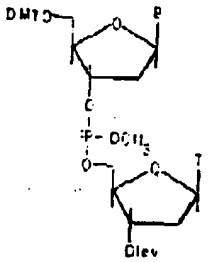
Department of Chemistry, University of Colorado, Boulder, Colorado 80309, U.S.A.

The development of a new class of nucleoside phosphites is described. These compounds are stable to normal laboratory conditions, are activated by mild acid treatment, and are observed to react essentially quantitatively with protected nucleosides.

A recent, key innovation in oligonucleotide synthesis was the introduction of the phosphite coupling approach by Letsinger and coworkers (1-3). This approach has been adapted to the synthesis of deoxyoligonucleotides (4-8), oligoribonucleotides (9-12), and nucleic acid analogs (13-15). Generally the approach involves the reaction of a suitably protected nucleoside, a difunctional phosphitylating agent such as methoxydichlorophosphine, and a second protected nucleoside. Mild oxidation using iodine in tetrahydrofuran, lutidine and water generates the natural internucleotide bond. By varying the oxidation procedure, phosphorus analogs such as selenophosphates (14), imidophosphates (14) and thiophosphates (14, 15) can be generated. A serious limitation of this methodology, however, has been the instability of the reactive intermediates (nucleoside phosphonomonochloridites or monotetrazolides) towards hydrolysis and air oxidation. This problem has been circumvented by either preparing the reactive species immediately prior to use or storing the active phosphite as a precipitate in hexanes at -20°C. We have recently solved this problem by synthesizing a new class of nucleoside phosphites that are easy to prepare by standard organochemical procedures, are stable under normal laboratory conditions to hydrolysis and air oxidation, and are stored as dry, stable powders. These key intermediates are N, N-dimethylaminophosphoramidites of the appropriately protected deoxynucleosides and are



Ia-d



IIIa-d

Ia, IIIa, B = 1-Thyminyl
Ib, IIIb, B = 1-(N-4-Benzoylcytosinyl)
Ic, IIIc, B = 9-(N-6-Benzoyladeninyl)
Id, IIId, B = 9-(N-2-Isobutyrylguaninyl)
lev = levulinyl
DMT = Di-p-anisylphenylmethyl

represented as compounds Ia-d. This communication outlines the synthesis, characterization, and reactivity of these phosphoramidites.

The synthesis of compounds Ia-d begins with the preparation of chloro-N, N-dimethylaminomethoxyphosphine [$CH_3O\ P(Cl)\ N(CH_3)_2$] which is used as a monofunctional phosphitylating agent. A 250 ml addition funnel was charged with 100 ml of precooled anhydrous ether (-78°C) and precooled (-78°C) anhydrous dimethylamine (45.9 g, 1.02 mol). The addition funnel was wrapped with aluminum foil containing dry ice in order to avoid evaporation of dimethylamine. This

solution was added dropwise at -15°C (ice-acetone bath) over 2 h to a mechanically stirred solution of methoxydichlorophosphine (16) (47.7 ml, 67.32 g., 0.51 mol) in 300 ml of anhydrous ether. The addition funnel was removed and the 1 L, three-necked round bottom flask was stoppered with serum caps tightened with copper wire. The suspension was mechanically stirred for 2 h at room temperature. The suspension was filtered and the amine hydrochloride salt was washed with 500 ml anhydrous ether. The filtrate and washings were combined and ether was distilled at atmospheric pressure. The residue was distilled under reduced pressure. The product was collected at 40-42°C @ 13 mm Hg and was isolated in 71% yield (51.1 g, 0.36 mol). d^{25} = 1.115 g/ml. ^{31}P-N.M.R., δ = -179.5 ppm ($CDCl_3$) with respect to internal 5% v/v aqueous H_3PO_4 standard. ^{1}H-N.M.R. doublet at 3.8 and 3.6 ppm J_{P-H} = 14 Hz (3H, OCH_3) and two singlets at 2.8 and 2.6 ppm (6H, $N(CH_3)_2$). The mass spectrum showed a parent peak at m/e = 141.

The key intermediates 1a-d were prepared by the following procedure. 5'-O-Di-*p*-anisylphenylmethyl nucleoside (1 mmol) was dissolved in 3 ml of dry, acid free chloroform and diisopropylethylamine (4 mmol) in a 10 ml reaction vessel preflushed with dry nitrogen. $[CH_3OP(Cl)N(CH_3)_2]$ (2 mmol) was added dropwise (30-60 sec) by syringe to the solution under nitrogen at room temperature. After 15 min the solution was transferred with 35 ml of ethyl acetate into a 125 ml separatory funnel. The solution was extracted four times with an aqueous, saturated solution of NaCl (80 ml). The organic phase was dried over anhydrous Na_2SO_4 and evaporated to a foam under reduced pressure. The foam was dissolved with toluene (10 ml) (Id was dissolved with 10 ml of ethyl acetate) and the solution was added dropwise to 50 ml of cold hexanes (-78°C) with vigorous stirring. The cold suspension was filtered and the white powder was washed with 75 ml of cold hexanes (-78°C). The white powder was dried under reduced pressure and stored under nitrogen. Isolated yields of compounds 1a-d were 90-94% (see Table I).

TABLE I

COMPOUND	δ-^{31}P (ppm) (Acetone-d_6)	δ-^{31}P (ppm) ($CDCl_3$)	ISOLATED YIELD (%)
1a	-146.0, -145.4	-147.7, -146.8	93, 95*
1b	-146.3, -145.5	-148.0, -147.0	92, 95*
1c	-146.1, -145.8	-147.4, -147.3	90, 98*
1d	-145.9, -145.7	-147.7, -147.2	90, 98*
IIIa	-139.6, -138.9	-140.8, -139.9	97**
IIIb	-139.6, -139.0	-140.6, -140.0	94**
IIIc	-139.7, -138.9	-141.0, -139.9	97**
IIId	-140.3, -140.2	-143.6, -141.9	93**

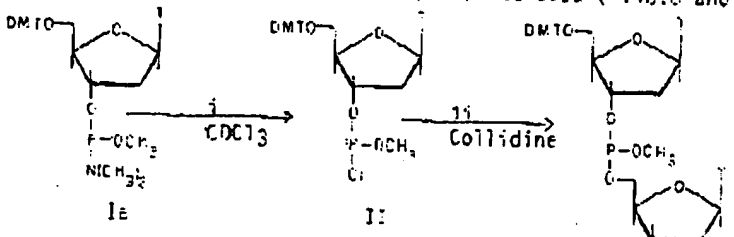
*Estimated purity from ^{31}P-N.M.R.

**Estimated yield from ^{31}P-N.M.R.

The purity of the products was checked by ^{31}P-N.M.R. Additionally, when analyzed by ^{31}P-N.M.R., these compounds were stable for at least a month when stored at room temperature under nitrogen. Furthermore, no significant amount of 3'-3' dinucleoside phosphite was detected by ^{31}P-N.M.R. (less than 4%). The low content of the 3'-3' dinucleoside phosphite was expected and represents a significant improvement over the original phosphite coupling procedure where a considerable amount of the unwanted 3'-3' dinucleoside phosphite was unavoidable (1-3, 9).

We have observed that mild acidic conditions can be used to activate 1a-d toward formation

of phosphite internucleotide bonds. These investigations were prompted by earlier research showing that aminophosphines can be protonated and therefore activated by acidic species (17-24). This activation process was initially monitored by ^{31}P-N.M.R. Thus, when N, N-dimethylaniline hyorochloride (1 mmol) in 0.5 ml of dry $CDCl_3$ was added at room temperature under nitrogen to Ia (0.5 mmol, -147.7 and -146.8 ppm) in 2 ml of dry, acid free $CDCl_3$ in a 10 mm N.M.R. tube, the chlorophosphite II (-167.2 ppm) was obtained in quantitative yield. Addition of 1.2 molar equivalents of 3'-O-levulinylthymidine (25) to the chlorophosphite II led to an essentially quantitative conversion to the dinucleoside phosphite IIIa (-140.8 and -139.9 ppm).



i, N, N-Dimethylaniline hydrochloride
ii, 3'-O-Levulinylthymidine

Evidence supporting the assignment of the active chlorophosphite II to the peak at -167.2 was independently obtained by reacting 5'-O-Di-p-anisylphenylmethylthymidine with excess methoxydichlorophosphine (-181.6 ppm) in the presence of collidine in $CDCl_3$. The major reaction product as monitored by ^{31}P-N.M.R. was localized at -167.2 ppm.

Of the various weak acids investigated as potential activating agents, 1H-tetrazole fulfills all requirements. The compound is a non-hygroscopic, commercially available solid that can be easily purified and dried in one step by sublimation at 110°C @ 0.05 mm Hg. Activation by 1H-tetrazole was also monitored by ^{31}P-N.M.R. Thus, Ia (0.5 mmol) and 3'-O-levulinylthymidine (0.6 mmol) were placed in a 10 mm N.M.R. tube and sublimed 1H-tetrazole (1.5 mmol) in 2.5 ml of dry acetonitrile-d_3 was added under nitrogen atmosphere. The ^{31}P-N.M.R. spectrum was immediately recorded and displayed a quantitative yield of IIIa. Similar results were also obtained when Ib, Ic and Id were reacted with 3'-O-levulinylthymidine. The appropriate chemical shifts of compounds Ia-d and IIIa-d with respect to internal 5% v/v aqueous H_3PO_4 standard are reported in Table I. Complete physical and analytical properties of these compounds will be reported elsewhere.

The applicability of these reagents to the synthesis of deoxyoligonucleotides on polymer supports was also tested. Trial experiments were completed by condensing compounds Ia-d with N-2-isobutyryldeoxyguanosine attached covalently to silica gel. Thus, N-2-isobutyryldeoxyguanosine (1 μmole) covalently attached to silica gel (20 mg) at the 3'-position, Ia (10 μmole), and 1H-tetrazole (50 μmole in 0.1 ml dry acetonitrile) were shaken for 20 min and the reaction was then quenched with aqueous lutidine. The same reaction sequence was completed with Ib, Ic and Id. After the usual oxidation and deprotection procedures (8), d(TpG), d(CpG), d(ApG) and d(GpG) were obtained in 100%, 98%, 94%, and 93% yield respectively (measured spectrometrically from the dimethoxytrityl cation using an extinction of 7×10^4 at 498 nm). These dinucleotides were completely degraded by snake venom phosphodiesterase and the appropriate nucleosides and

nucleotides were obtained in the proper ratios (monitored via high pressure liquid chromatography analysis of snake venom phosphodiesterase hydrolysates).

The N, N-dimethylamino phosphines 1a-d therefore display tremendous potential in oligodeoxynucleotide synthesis. These compounds are easy to prepare and are stable to normal laboratory conditions. They are readily activated via protonation and condense with appropriate nucleosides to form internucleotide bonds in very high yields.

Acknowledgements

This is paper 5 in a series on Nucleotide Chemistry. This work was supported by grants from the National Institutes of Health (GM21120 and GM25680). S. Beaucage acknowledges a postdoctoral fellowship from the Natural Sciences and Engineering Research Council of Canada. M. Caruthers was supported by a Career Development Award from the National Institutes of Health (1 K04 GM00076).

References

1. R. L. Letsinger, J. L. Finnan, G. A. Heavner, W. B. Lunsford, *J. Am. Chem. Soc.* 97, 3278-3279 (1975).
2. R. L. Letsinger and W. B. Lunsford, *J. Am. Chem. Soc.* 98, 3655-3661 (1976).
3. R. L. Letsinger, J. L. Finnan, S. A. Jacobs, B. A. Juodka and A. K. Varshney, *Proc. Int. Conf. Transfer RNA*, Poznan, Poland, October 1976, pp 145-155.
4. M. D. Matteucci and M. H. Caruthers, *Tetrahedron Lett.*, 21, 719-722 (1980).
5. J. L. Finnan, A. Varshney and R. L. Letsinger, *Nucl. Acids Res. Symposium Series No. 7*, 133-145 (1980).
6. M. H. Caruthers, *Proc. of IUPAC International Symposium on Macromolecules*, Florence, Italy, September 1980, Pergamon Press, Oxford, England.
7. M. H. Caruthers, S. L. Beaucage, J. W. Efcavitch, E. F. Fisher, M. D. Matteucci and Y. Stabinsky, *Nucl. Acids Res. Symposium Series No. 7*, 215-223 (1980).
8. M. D. Matteucci and M. H. Caruthers, *J. Am. Chem. Soc.*, In press.
9. G. W. Daub and E. E. van Tamelen, *J. Am. Chem. Soc.* 99, 3526-3528 (1977).
10. K. K. Ogilvie and M. J. Nemer, *Can. J. Chem.* 58, 1389-1397 (1980) and references therein. See also K. K. Ogilvie, N. Y. Theriault, J. M. Seifert, R. T. Pon and M. J. Nemer, *Can J. Chem.* 58, 2686-2693 (1980).
11. K. K. Ogilvie, M. J. Nemer, N. Theriault, R. Pon and J. M. Seifert, *Nucl. Acids Res. Symposium Series No. 7*, 147-150 (1980).
12. K. K. Ogilvie and M. J. Nemer, *Tetrahedron Lett.* 21, 4159-4162 (1980).
13. B. P. Melnick, J. L. Finnan and R. L. Letsinger, *J. Org. Chem.* 45, 2715-2716 (1980).
14. K. K. Ogilvie and M. J. Nemer, *Tetrahedron Lett.* 21, 4145-4148 (1980); ibid., 4149-4152 (1980); ibid., 4153-4154 (1980).
15. P. M. J. Burgers and F. Eckstein, *Tetrahedron Lett.*, 3835-3838 (1978).
16. D. R. Martin and P. J. Pizzolato, *J. Am. Chem. Soc.* 72, 4584-4586 (1950).
17. H. Nöth and H. J. Vetter, *Chem. Ber.* 96, 1109-1118 (1963).
18. E. E. Nifant'ev, N. L. Ivanova and N. K. Bliznyuk, *Zh. Obschei Khim.* 36, 765 (1966).
19. E. E. Nifant'ev, N. L. Ivanova and I. V. Fursenko, *Zh. Obschei Khim.* 39, 854-856 (1969).
20. V. N. Eliseenkov, A. N. Pudovik, S. G. Fattakhov and N. A. Serkina, *Zh. Obschei Khim.* 40, 498 (1970).
21. V. P. Evdakov, V. P. Beketov and V. I. Svergun, *Zh. Obschei Khim.* 43, 55-59 (1973).
22. A. N. Pudovik, E. S. Batyeva, E. N. Ofitserov and V. A. Al'fonsov, *Zh. Obschei. Khim.* 45, 2338-2339 (1975).
23. E. S. Batyeva, V. A. Al'fonsov, G. U. Zamaletdinova and A. N. Pudovik, *Zh. Obschei. Khim.* 46, 2204-2207 (1976).
24. V. A. Al'fonsov, V. A. Kharlamov, E. S. Batyeva, T. Kh. Gazizov and A. N. Pudovik, *Iz. Akad. Nauk. SSSR Ser. Khim.*, 2194-2196 (1977).
25. A. Hessner, G. Strand, M. Rubinstein, A. Patchornik, *J. Am. Chem. Soc.* 97, 1614-1615 (1975).

(Received in USA 12 February 1981)